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PRINCIPAL INVESTIGATOR: Harold H. Sandstead, M.D.

CONTRACTING ORGANIZATION: University of Texas
Galveston, Texas 77550

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Harold H. Sandstead, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas
Galveston, Texas 77550

E-MAIL:
hsandste@utmb.edu

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13. ABSTRACT ***(Maximum 200 Words)***

Primary Hypothesis: Repletion of mild zinc (Zn) & iron (Fe) deficiencies will improve neuropsychological performance of women. Design: This is a 16 week double-blind stratified randomized controlled treatment trial with a treatment cross-over at 8 weeks. The subjects are 60 Zn & Fe deficient (D) & 20 normal (N) premenopausal women. Other potentially limiting nutrients are repleted before collection of baseline data. Treatments are: micronutrients (M) alone, given to 20 D & 20 N subjects for 16 weeks with a pseudo-cross-over at 8 weeks; 30 mg Zn with M and 30 mg Fe with M, each given to 40 D subjects for 8 weeks followed by a cross over to the other treatment. Outcomes include indices of Zn & Fe status, lean body mass (LBM), other indices that might be affected by Zn status, and neuropsychological performance. Results: The treatment trial was completed. Completion of laboratory and data analysis is in process

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Harold L. Swinstead, MD 2/6/2000

PI - Signature Date

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Introduction

Subject: The relationship of zinc and iron nutriture to neuropsychological performance of women.

Purpose: This project tests the hypothesis: Repletion of mild zinc and iron deficiencies will improve neuropsychological performance of premenopausal women.

Scope: The three major components of the project are:

- Premenopausal women judged likely to be deficient in zinc and iron are identified by food frequency history and concentration of serum ferritin.
- Zinc status is determined by plasma, white blood cell, urine, and hair zinc concentrations, and zinc kinetics. Iron status is determined by serum ferritin and iron concentrations, the percent iron saturation of transferrin, hemoglobin concentration and red blood cell indices.
- Effects of zinc and iron repletion on neuropsychological performance are determined through a double-blind stratified randomized controlled treatment trial with a crossover design and computerized measurement of neuropsychological function before and after treatment.

Statement of Work

This project tests the hypothesis: Repletion of mild zinc and iron deficiencies will improve neuropsychological performance of premenopausal women.

The design is a double blind stratified randomized controlled treatment trial with a cross-over of treatments. Sixty zinc (Zn) and iron (Fe) deficient and 20 normal control subjects are studied. Before the treatment trial begins all subjects are repleted with a mixture of micronutrients (M) based on the Recommended Dietary Allowances for 7 days so as to replete deficiencies that might interfere with responses to the experimental repletions. Then, serving as controls, 20 deficient subjects and 20 normal subjects are given M for 16 weeks. After the first 8 weeks they undergo a pseudo-cross over of treatment. Of the 40 remaining deficient subjects, 20 are repleted with 30 mg Zn with M (ZnM), and 20 are repleted with 30 mg Fe with M (FeM) for 8 weeks. Then the treatments of these latter two groups are switched so as to accomplish a crossover. Neuropsychological performance is measured at baseline and after 8 and 16 weeks of treatment. Individual and combined effects of Zn and Fe, and unique effects related to the order of treatments are determined. Secondary outcomes include improved methods for measurement of Zn tracers by inductively couple plasma-mass spectroscopy ICP-MS; measurement of zinc kinetics; measurement of relationships between zinc kinetics and lean body mass; measurement of relationships between changes in Zn and Fe and other aspects of metabolism such as plasma pyridoxal-5 phosphate, folate, cyanocobalmine, hydroxyproline and free amino acids.

Project Time Line (from the grant application, edited and status indicated):

0-90 days	Hire personnel Purchase initial equipment and supplies---------Minimal needs continue Advertise project; interview respondents----------616 total, 200 this year Screen respondents----------262 total, 112 this year
Year 1.	Goal: enroll 30 subjects in the treatment trial 5 subjects qualify 5 subjects enrolled Great difficulty with recruitment and retention, cause unclear
Year 2	Goal: enroll 30 subjects in the treatment trial 50 subjects qualify 32 subjects enrolled 22 complete the first phase of the treatment trial 15 subjects complete the study 8 subjects resigned Assistant resigned, new assistant hired, recruitment improved Subject retention impaired by the length of the protocol. With permission, Zn kinetic studies were stopped after 52 subjects.
Year 3	Goal: enroll 20 subjects in the study 52 subjects qualified 45 subjects enrolled 37 completed the first phase of the treatment trial 34 subjects completed the study 49 total subjects have completed the study 7 subjects are at present part way though the treatment trial 6 subjects resigned New administrative assistant hired, good recruitment continues Permission was granted for study continuation
Year 4	Goal: complete enrollment. 119 women were interviewed by telephone. 63 were screened. 27 met the study criteria. 35 entered the randomized controlled trial. 34 completed the first phase of the study. 36 completed the study. Thus the planned number of subjects was studied. Permission was granted for study continuation.
Year 5	Complete laboratory analysis and data evaluation. Prepare final reports.

Subjects Time Line (with Notes):

d 1	Telephone interview respondents to advertisements -------119 this year
d 7	Screen respondents that qualify for the project ----------63 this year
	Medical history ----------by our staff
	Food frequency history ----------by our staff
	Physical Examination ----------by our staff
	Screening blood chemistries, CBC, serum ferritin provided by the NIH sponsored Clinical Research Center
d 14	Select subjects ----------27 this year
	Treat latent deficiencies with micronutrients before baseline measurements of outcomes. The duration (about 7-10 days) of treatment depends on stage of menstrual cycle.
d 24	Orient the subjects to the neuropsychological tasks
	Randomize the subjects to the treatments
	Measure baseline bioelectrical Impedance, taste acuity and other indices related to Zn status
~d 30	Measure baseline neuropsychological outcomes on day 8-12 of menstrual cycle ----------35 this year
	Begin treatment
~d 90	After 8 weeks of treatment repeat measurements of outcomes on day 8-12 of menstrual cycle ----------34 this year
	Cross-over the zinc and iron treatment groups and pseudo-cross-over the control groups
~d 150	After 16 weeks of treatment measure outcomes on day 8-12 of menstrual cycle ----------36 this year
	Thank subjects for participation
	Give subjects a copy of their medical evaluation
	Give subjects nutrition information
	Pay the final compensation
Later	Send subjects copies of published reports

Methods:

Subject Recruitment: Advertisements are posted on bulletin boards at UTMB and surrounding Colleges, in news letters and newspapers, and on the Internet. Respondents are screened by telephone interview. Potential candidates are invited for detailed evaluation by medical and dietary history, physical examination and laboratory assessment, and determination of Fe status. Individuals with serum ferritin <20μg/mL or >30μg/mL who are otherwise normal were offered an opportunity to volunteer for the study.

Assessment of Zinc Status: Plasma zinc concentrations are measured by Atomic Absorption Spectrometry (AAS) (1). Granulocyte, lymphocyte and platelet zinc are measured by AAS (2, 3). Hair zinc is measured by AAS (4, 5).

Assessment of Iron Status: Iron status is assessed by serum ferritin (6, 7), serum iron, percent saturation of transferrin, and hemoglobin concentration (8).

Other Indices Possibly Affected by Zinc Nutriture: An electrogustometer (Rion TR-06) developed by Tomita et al (9) is used to measure the electrical threshold of taste buds. A circular, 5mm in diameter, stainless steel electrode was used. Subject were comfortably sitting and familiar with the difference between the electrical stimulation and the tactile sensation of the electrode. A neutral electrode was applied to the neck and the voltage adjusted to 10 dB above the expected threshold. Subjects were asked to identify when they experienced an electrical sensation by pressing a buzzer. Areas of the tongue, 2 cm from the midline, on the left and right side of the anterior and posterior tongue (circumvallate papillae) were assessed. Threshold are determined by increasing the stimulation current starting from zero. Taste acuity is measured before and after treatment. Taste acuity is also measured by the filter paper disc method. A drop of a solution of either sweet (sucrose), salty (sodium chloride), sour (tartaric acid), and bitter (quinine hydrochloride) was placed on a small 5 mm paper disc on the same areas of the tongue as described for the electrogustometer, left for 3 seconds and then removed. The mouth is then rinsed with water. The subject is asked to identify the taste quality. The level at which the taste quality is correctly identified was the threshold. Previous experiments found Zn essential for taste (10-12).

β-hydroxybutyrate (13) is measured in serum using a kit (Procedure No. 310-UV, Sigma Diagnostics, PO Box 14508, St. Louis, MO). The purpose of these measurements was to determine if zinc nutriture, at the levels observed in these subjects, had a practical effect on fat metabolism, as was observed in zinc deprived rats (14).

Since the start of the project and at not cost to the project, blood from subjects is assayed in plasma and erythrocyte folate using *L. casei* (15, 16) by T. Tamura, MD, of the University of Alabama Department of Nutrition Sciences. He also measured plasma cyanocobalamine concentrations using a Ciba-Corning (Medfield, MA) Radioassay kit, plasma pyridoxal-5-phosphate concentrations by the method of Camp et al. (17), and plasma homocysteine concentrations by the method of Tamura (18). The purpose of these measurements is to determine if Zn nutriture, at the levels present in our subjects, has a practical effect on these important indices, as was suggested by other experiments (19-22).

Amino acids are measured by Richard Fritz, PhD of the Department of Human Biological Chemistry and Genetics at UTMB in serum and urine baseline and follow-up samples from 50 subjects. The purpose of these measurements is to determine if Zn nutriture, at the level observed in our subjects, has a practical effect on amino acid metabolism, as was observed in rats (23, 24).

David Simmons, PhD of the UTMB Department of Orthopedics measures 24 hr urine cross linked n-telopeptides for type I collagen in baseline samples, after the first period of treatment, and after the second period of treatment; and serum osteocalcin in samples from each phase of the repletion study. The purpose of these measurements

is to determine if Zn nutriture, at the level observed in our subjects, has a practical effect on bone metabolism, as was suggested by other experiments (25-27).

Douglas Goeger, PhD, of the UTMB Department of Preventive Medicine and Community Health measures metabolism of the substrate chlorzoxazone to determine if Zn nutriture affects activity of a Zn mediated cytochrome P450 mixed function oxidase. After an over-night fast subjects were given 500 mg chlorzoxazone orally and the fasting continued for 2 hours. Then 5 mL of blood was drawn into an EDTA containing vacutainer, and the plasma separated and stored at -20° C until analysis of the ratio of chlorzoxazone and 6-hydroxychlorzoxazone is determined (28). The ratio at this time point is significantly correlated to area under the curve ratios for 6-hydroxychlorzoxazone and chlorzoxazone and is less invasive than multiple blood samples needed to determine area under the curve (29). This procedure has been used successfully for detecting changes in chlorzoxazone (29). The metabolism of chlorzoxazone to 6-hydroxychlorzoxazone can vary 4 to 5-fold between individuals, and no age or sex-related effects are apparent (30). This study shows if Zn nutriture, in the range observed in these subjects, has a practical effect on this important enzyme, as was suggested by experiments in rats (31).

Zinc Kinetics: Serum and urine collected for measurement of zinc kinetics are analyzed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) by modification of published methods (32). These measurements show if indices of zinc kinetics are related to serum ferritin concentrations, as our previous findings suggest (33); and if indices of zinc kinetics are related to indices of neuropsychological performance at baseline and/or after treatment.

Effect of Zinc and Iron Repletion on Outcomes: Neuropsychological performance indices is measured on the 8-12th day of the menstrual cycle before and after the experimental treatments. Data from this phase of the project is being analyzed.

Results and Discussion:

We requested and received permission to continue the study for one additional year (with no additional money).

Subject Recruitment: was successful

Zinc Status: More than half of subjects had serum Zn concentrations near 700 μg/L, the empirical lower limit of normal (34). While plasma zinc is an insensative indicator of zinc status (35) this findings is consistent with recent dietary intake of Zn being marginal. The finding suggests our selection criteria were appropriate.

Leukocyte and platelet zinc concentrations were measured by the method of Beck (3) in baseline (after repletion of other potentially limiting nutrients) were quite variable. Though others report these indices are useful indicators of Zn status, reference ranges in the literature are wide. This may be because of technical difficulties with the isolation

and assay of white blood cells. Our findings were generally within the reference ranges. We will compare our WBC Zn data with indicators of cognitive function before and after Zn repletion.

Hair Zn concentrations of most subjects were within the reference range.

Other Indices Possibly Affected by Zinc Nutriture:

Final analysis of the taste acuity data is in the process of completion.

Plasma beta-hydroxybutyrate concentrations were within the reference range.

Analysis of remaining samples is being done by our collaborator T. Tamura at the University of Alabama. To date findings continue to be similar to those reported last year. Plasma homocysteine, plasma and erythrocyte folate and plasma cobalamine were within the normal range at screening and after repletion of other potentially limiting micronutrients. There was no change in the concentrations of these indices after repletion. In contrast, plasma pyridoxal-5-phosphate (PLP) concentration was marginal to low in many subjects and was resistant to change after repletion in many subjects. When analysis are complete we will relate the findings to the treatment groups. We suspect Zn repletion will be associate d with an increase in PLP because pyridoxal kinase uses Zn-ATP as a substrate (21).

The analysis of plasma and urine samples for amino acids is in progress.

The analysis of samples for assessment of bone metabolism is in progress.

No effects of zinc nutriture on metabolism of blood cytochrome P450 activity were detected.

Zinc Kinetics:
Preliminary analysis did not find a relation between plasma Zn disappearance and serum ferritin. This is in contrast to our earlier finding of a highly significant inverse relationship (33). Reanalysis of some samples has been completed and we are reevaluating the plasma Zn disappearance data.

Effects of Zn or Fe on Neuropsychological Performance:
The double blind randomized controlled repletion trial is progressing.

Conclusions:
The randomized controlled trial was completed. Final laboratory and data analysis are in process.

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Presentations:

Abstract presented to the American Society for Nutrition Sciences at Experimental Biology '99, Washington, DC, April 17-21, 1999.

1)Determination of the Rapidly Exchangeable Zinc Pool in Humans by a Random Urine Specimen. K. Yokoi, N. Egger, V.M. Sadagopa Ramanujam, N.W. Alcock, , H.H. Dayal, H.H. Sandstead.

Publications:

Ramanujam VMS, Yokoi K, Egger NG, Dayal HH, Alcock NW Sandstead HH, Polyatomics in Zinc Isotope Ratio Analysis of Plasma Samples by Inductively Coupled Plasma-Mass Spectrometry and Applicability of Nonextracted Samples for Zinc Kinetics. Biological Trace Element Research 1999; 68: 143-58.

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PART I
ABSTRACTS 1.1-486.12

The American Physiological Society
American Society for Pharmacology and Experimental Therapeutics
American Society for Investigative Pathology
American Society for Nutritional Sciences
The American Association of Immunologists
American Association of Anatomists

The Biomedical Engineering Society
Society for Experimental Biology and Medicine
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The Microcirculatory Society
North American Vascular Biology Organization
Society for International Nutrition Research
American Society for Clinical Nutrition

Association of Medical Laboratory Immunologists
American Society for Histocompatibility and Immunogenetics
International Society of Developmental and Comparative Immunology
International Society for Interferon and Cytokine Research
International Society for NeuroImmunoModulation
International Society of Neuroimmunology
Society for Leukocyte Biology
Society for Mucosal Immunology
American Federation for Aging Research
Clinical Immunology Society
American Association of Veterinary Immunologists
Society of Natural Immunity

448.3

DETERMINATION OF THE RAPIDLY EXCHANGEABLE ZINC POOL IN HUMANS BY A RANDOM URINE SPECIMEN.

K. Yokoi, N. G. Egger, V. M. Sadagopa Ramanujam, N. W. Alcock, H. H. Daval and H. H. Sandstead. University of Texas Medical Branch, Galveston, TX 77555 and Jichi Medical School, Tochigi, Japan.

The rapidly exchangeable zinc pool (EZP) is considered to represent metabolically active zinc and is determined from zinc kinetic models that necessitate tedious collection of samples over several days. We found the 24-h spot plasma pool as an estimate of EZP. We further investigated the usefulness of just a spot urine sample obtained 24-h after iv ^{67}Zn tracer for estimation of EZP (24-h spot urine pool). Twenty-nine healthy women were studied (age ranged from 19-39 years, body weight from 47-85.5 kg, body height from 1.49-1.77 m, body mass index from 17-36 kg/m^2). Zn isotope ratios in plasma and urine were measured by inductively coupled plasma mass spectrometry and the tracer-to-tracee ratios (TTR) were calculated. The dose of ^{67}Zn tracer was divided by TTR to obtain spot zinc pool sizes. Plasma zinc was measured by atomic absorption spectrometry. The 24-h spot plasma Zn pool ranged from 103-240 mg, the 24-h spot urine Zn pool from 79-222 mg, and plasma Zn from 625-939 ng/mL. The 24-h spot urine pool was found to be significantly and positively correlated with the 24-h spot plasma pool ($r=0.91$, $p<0.01$), body weight ($r=0.66$, $p<0.01$) and lean body mass ($r=0.73$, $p<0.01$). The ratio of the 24-h spot urine pool to the 24-h spot plasma pool ranged from 0.64-0.98. These results suggest that the 24-h spot urine can be used to estimate EZP in place of the 24-h spot plasma. *(Supported by a Department of Defense grant, DAMDB 17-95-C-5112).*

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Polyatomics in Zinc Isotope Ratio Analysis of Plasma Samples by Inductively Coupled Plasma–Mass Spectrometry and Applicability of Nonextracted Samples for Zinc Kinetics

V. M. SADAGOPA RAMANUJAM,*[,1] K. YOKOI,[2] N. G. EGGER,[1] H. H. DAYAL,[1] N. W. ALCOCK,[1] AND H. H. SANDSTEAD[1]

[1]*Department of Preventive Medicine and Community Health, University of Texas Medical Branch, Galveston, TX 77555-1109; and* [2]*Department of Environmental Health, Jichi Medical School, Yakushiji, Minamikawachi-machi, Tochigi-Ken 329-04, Japan*

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ABSTRACT

Inductively coupled plasma–mass spectrometry (ICP-MS) is a powerful tool for both quantitative multielement analyses of inorganic elements and measurement of isotope ratios (IRs). The main disadvantage of this technique is the existence of polyatomic isobaric interferences at some key masses. Zinc has been investigated for such potential interferences in serum or plasma. The Zn isotopes, ^{66}Zn and ^{68}Zn, have no apparent interferences, but $^{32}S^{16}O_2$ and $^{32}S_2$ are isobaric with ^{64}Zn. The possible effects of S and other major components of blood plasma—Na, K, Cl, P, Ca—on Zn IRs were investigated using a series of mineral solutions which simulated human plasma with respect to these elements. The mixture of all mineral elements interfered only with ^{64}Zn (6.66 ng/mL) and ^{70}Zn (8.51 ng/mL). Interferences to ^{66}Zn, ^{67}Zn, and ^{68}Zn were minimal containing 0.90, 0.94, and 0.39 ng/mL, respectively. The copresence of Na or S shifted $^{35}Cl^{16}O_2$ (atomic mass 67 coming from Cl

*Author to whom all correspondence and reprint requests should be addressed.

solution) to $^{35}Cl_2$ which reduced the contribution to ^{67}Zn. The hypothesis that Zn IRs obtained from plasma at various intervals after the intravenous administration of enriched ^{67}Zn to humans would reflect those obtained after extraction of Zn was therefore tested. To compare the two pretreatment methods, "extraction" versus "nonextraction," specimens were collected from 10 human subjects at intervals of 5 min to 24 h postinjection, and in 4 subjects from 5 min to 9 d postinjection. Two separate aliquots of plasma from each time-point were dried and digested with hydrogen peroxide, and the residue dissolved in nitric acid. One specimen was subjected to zinc extraction using ammonium diethyldithiocarbamate chelate followed by back extraction into nitric acid. The matching aliquot received no further pretreatment. The normalized IRs obtained from $^{67}Zn/^{66}Zn$ and $^{67}Zn/^{68}Zn$ in both the "extracted" and "nonextracted" samples agreed well (r^2 = 0.976 and r^2 = 0.985, respectively) compared to those from other ratios (r^2 = 0.838 for $^{67}Zn/^{64}Zn$ and r^2 = 0.747 for $^{67}Zn/^{70}Zn$). Considering the minimum possibility of isobaric interferences in plasma samples, $^{67}Zn/^{68}Zn$ obtained from "nonextracted" samples is sufficient for routine Zn kinetic analysis by ICP-MS.

Index Entries: Enriched ^{67}Zn isotope; extraction, nonextraction; inductively coupled plasma–mass spectrometry (ICP-MS); isotope ratios (IRs); Zn kinetics; Zn turnover rate (TR); exchangeable Zn pool (EZP).

INTRODUCTION

For the assessment of Zn nutriture, determinations of plasma or serum Zn concentrations are used extensively in clinical practice, with normal plasma or serum Zn concentrations generally considered to be 700–1200 μg/L. However, many physiological factors *(1)* influence plasma zinc. Plasma or serum zinc concentrations vary with stress conditions unassociated with Zn deficiency and the zinc content of accessible tissues does not provide a reliable index of its status. Isotopic techniques seem to provide an answer to this problem.

Radioisotopes of Zn have been used to develop complex mathematical models which describe Zn kinetics under various conditions in man and laboratory animals *(2–5)*. Such models have been used to identify sites of regulation of Zn metabolism and calculate the size and turnover rate of body Zn pools. A simpler model describing Zn kinetics over a short time period (90 min) has been developed using ^{65}Zn in the rat *(6)*. Using this model, it was shown that a rapidly exchanging pool of Zn is responsive to changes in dietary Zn intake, becoming significantly depleted in animals maintained on a Zn-deficient diet. ^{65}Zn has a biological half-life of 500 d *(7)*; hence, its applicability in the study of Zn metabolism in animals or humans is limited.

A potential approach to the study of relationships between dietary Zn supply and body status is the measurement of plasma Zn kinetics following an intravenous injection (iv) of a nonradioactive stable Zn isotope. Stable isotopes offer an advantage over radioisotopes in that there is no radiation exposure of the subjects. Stable isotopes offer a clear advantage over radioisotopes in that they occur naturally and, hence, have been used to study various aspects of zinc metabolism in humans *(8–12)*, although only Jackson et al. *(13,14)* used ^{67}Zn to examine Zn turnover rates. Several instrumental techniques have been reported for the determination of isotope ratios (IRs) of Zn, including neutron activation analysis *(4,15–17)* and mass spectral methods such as thermal ionization *(18)*, fast atom bombardment *(12,19–21)*, and inductively coupled plasma *(22–24)*.

Recently, we *(25,26)* and others *(6,14,23)* have shown that stable isotopes of Zn can be successfully used to measure Zn turnover rates (TR) and exchangeable Zn pools (EZP), which are responsive to changes in Zn status. The availability of and ability to measure stable isotopes of Zn by mass spectrometry make this a viable technique for Zn metabolic studies. Studies from our laboratory indicate that inductively coupled plasma–mass spectrometry (ICP-MS) provides sensitive and reliable detection of the isotopes of Zn.

The stable isotope kinetics methodology for the determination of Zn status involves (i) the use of an intravenously administered Zn-67 stable isotope tracer and collection of blood at various time points, and (ii) determination of isotope ratios $^{67}Zn/^{64}Zn$, $^{67}Zn/^{66}Zn$, $^{67}Zn/^{68}Zn$, and $^{67}Zn/^{70}Zn$ at each time-point using ICP-MS. The data are used to calculate Zn disappearance and turnover rates and the exchangeable Zn pools after injection. The findings are related to biochemical and physiological indices of functions in order to establish zinc status.

The isolation of Zn from samples by an extraction procedure has usually been performed to obtain accurate results for mass spectrometric analysis *(12,22,25,26)*. The extraction requires a number of procedural steps, which limits application to large numbers of specimens. Hence, potential interferences from nonextracted specimens have been explored. The major matrix elements in human blood plasma or serum samples are sodium (Na, 3130–3370 mg/L), chloride (Cl^-, 2940–4120 mg/L), and sulfur (S, 1120–1270 mg/L) *(27)*. Theoretically, there are no apparent major isobaric interferences for ^{66}Zn and ^{68}Zn in blood plasma, although $^{32}S^{16}O_2$ and $^{32}S_2$ overlap ^{64}Zn. Therefore, we wanted to test this theory by conducting a detailed study of comparing the isotope ratios obtained from several sets of "extracted" and "nonextracted" plasma samples. In this project, we compared the four different isotope ratios obtained from Zn-extracted and Zn-nonextracted digested (using hydrogen peroxide) plasma samples in order to determine whether the simple "nonextraction" procedure is applicable for routine Zn isotope ratio analysis for kinetic studies in humans.

EXPERIMENTAL

Chemicals, Reagents, and Supplies

The enriched stable isotope ^{67}Zn (as oxide, purity 93.11%) was purchased from Oak Ridge National Laboratory (Martin Marietta Energy Systems, Inc., Oak Ridge, TN, USA). Hydrogen peroxide (30%, ultrapure), nitric acid (ultrapure double-distilled from Vycor), ammonium hydroxide (high-purity grade), hydrochloric acid (ACS grade), and sulfuric acid (ACS grade) were purchased from GFS Chemicals (Columbus, OH, USA). Hydrochloric acid (suprapure grade) was obtained from EM Science (Gibbstown, NJ, USA).

Sodium nitrate, diammonium monohydrogenphosphate, and calcium carbonate (Baker analyzed chemical grades) were purchased from Baker (Phillipsburg, NJ, USA). Potassium nitrate (ACS grade) was obtained from Fisher Chemicals (Houston, TX). Carbon tetrachloride (ACS grade), 2,6-dinitrophenol, and Zn and yttrium reference standard solutions were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Diethyl ammonium diethyl dithiocarbamate was purchased from Tokyo Casei Co. (Tokyo, Japan).

Deionized water was prepared using a Milli-Q System (Millipore Corp., Milford, MA, USA). Monovette syringes containing lithium heparin (10 U/mL blood) used for blood collections and polypropylene sterile tubes (29 × 114-mm size with red caps) free from Zn contamination used for plasma digestions were purchased from Sarstedt Inc. (Newton, NC, USA). Disposable Falcon polypropylene tubes (15-mL capacity) used for preparing the final ICP-MS digestate solutions and absolute ethanol used to dissolve the 2,6-dinitrophenol indicator were purchased from Fisher Scientific Co. (Pittsburgh, PA, USA). The carbon tetrachloride extraction of Zn from the digestates were carried out in hydrochloric-acid (10%)-washed borosilicate glass tubes (Kimax Inc., Toledo, OH, USA).

Human Subjects and Zinc Kinetics

Five healthy men and one woman living in Galveston, Texas were the subjects for the 9-d observation. Eleven women in apparent good health who were participating in a study of effects of zinc and iron status on brain function participated in a 24-h observation study. This project was approved by the Institutional Review Board of the University of Texas Medical Branch (UTMB) and written consent was obtained from all subjects. The disappearance rate for ^{67}Zn from blood plasma, turnover rate, and the exchangeable Zn pool sizes were measured using the procedures well established in our laboratory *(25,26,28–30)*.

Zinc kinetics were measured using ^{67}Zn (natural abundance 4.11%; enrichment, 93.11%) chloride which was prepared from ^{67}Zn oxide by dissolving 59.52 mg in a few drops of concentrated hydrochloric acid

(ACS grade), and heating it to dryness on a hot plate. The synthesized chloride was dissolved in saline (12 mL, corresponds to 0.5 mL = 2 mg ^{67}Zn) aliquots of 0.5 mL, sterilized by passing the solution through Millipore (Fisher Scientific Co., Houston, TX) filter (0.2 μ*M* pore size) into glass vials containing 10.0 mL saline. Several of these vials (1 per 10 vials) were randomly selected and tested for sterility (Department of Clinical Microbiology and Immunology, UTMB) and pyrogenicity (Scientific Associates Inc., St. Louis, MO).

Subjects were admitted to the General Clinical Research Center at UTMB for administration of ^{67}Zn and collection of blood samples. The diet was limited in bioavailable zinc. At 7:00 AM, after the subject had fasted at least 12 h, short Teflon catheters connected to a three-way stop cock were placed in each antecubital vein and kept open by 0.9% saline solution. After 30 min, a blood sample was taken to establish the baseline $^{67}Zn/^{68}Zn$ ratio.

The ^{67}Zn tracer—2 mg in 10.5 mL saline further diluted to 30 mL in saline—was then administered over 3 min (timed by stopwatch). The line was flushed rapidly with saline for 30 s. Blood samples were then collected from the opposite arm starting 5 min after completion of the ^{67}Zn administration. Additional samples were collected at 5, 15, 30, 40, 50, 60, 90 min, and 2, 6, 12 h, and 1, 2, 3, 5, 7, and 9 d later. The 9-d and 1-d samples were collected from 4 and 10 subjects, respectively. Blood samples were placed in an ice chest after collection and delivered to the laboratory for processing. The blood samples were centrifuged at 2000*g* for 20 min and the plasma layers were transferred to polypropylene tubes and stored in a freezer (–70°C) until ready to use.

Digestion of Plasma and Extraction of Zinc

Sample digestion was based on the method of Alcock *(31)*. Duplicate aliquots of plasma were measured out in 50 mL polypropylene tubes, kept overnight at –70°C, transferred to a freeze-drier and lyophilized overnight, further dried for 8 hours at 80°C in an oven, and digested with 30% hydrogen peroxide (2 aliquots of 5 and 7 mL, high purity grade, GFS Chemicals) for 2 days at 85–90°C. The white ash was dissolved in 1.5 mL 1.2 M Ultrapure nitric acid. Several (4 tubes/batch) hydrogen peroxide blanks run throughout the entire procedure were used to calculate ICP-MS IR blank subtractions.

Extraction

The extraction of Zn was based on the method of Serfass et al. *(22)*, modified by Yokoi et al. *(26)*. After digestion of the plasma, the ash was dissolved in 1.5 mL of 1.2*M* nitric acid and the solution transferred to a 20-mL acid-washed borosilicate tube using the Zn-free polyethylene transfer pipet, followed by two washes with deionized water. One drop (40 μL) of 0.1% 2,6-dinitrophenol in 50% ethanol was added to the solution as a pH indicator. Dilute ammonium hydroxide was added in drops

with shaking the tube to bring the pH to 2.5 (indicated by the color change to yellow). One milliliter of 0.25% diethyl ammonium diethyl dithiocarbamate in carbon tetrachloride was added, the tube closed tight with a Teflon-lined cap, and the contents shaken vigorously for 2 min. Each tube was allowed to stand until separation of the acid and carbon tetrachloride layers was complete.

The carbon tetrachloride layer containing chelated zinc was transferred to another glass tube carefully, using the acid-washed glass Pasteur pipet, the Zn-chelate decomposed with 1 mL of 1.2*M* nitric acid, and the Zn back-extracted into the acid by vigorously shaking the tube. The back-extraction of Zn was usually indicated by the transfer of yellow color into the acid layer, followed by its disappearance. If such a transfer did not occur immediately, the solution was allowed to stand for 1 h and shaken again to complete the decomposition and transfer steps. The top acid layer was then transferred to another glass tube and the solution heated overnight at 80°C to remove traces of CCl_4; it was made up to 10 mL with Milli-Q deionized water after adding yttrium internal standard (100 µL of 5-mg/L solution in 1% nitric acid). Batches of 12–20 tubes were processed at one time. Yttrium was chosen as the internal standard because it has only one natural isotope, 89 amu, which gives abundant ICP-MS signals and is closer to Zn isotopic masses.

Nonextraction

After digestion of the plasma, the ash was dissolved in 1.5 mL of 1.2*M* nitric acid and each solution transferred to a polypropylene falcon tube followed by two washes with Milli-Q deionized water. The yttrium internal standard and Milli-Q water were directly added to the digestate and made up to 10 mL with water.

Solutions for the Measurement of ICP-MS Interferents

Because the preparation of human plasma for isotope ratio ICP-MS analysis involves digestion by hydrogen peroxide and solubilization of the obtained white ash with nitric acid, the main added matrix elements in the well-digested solution should only be hydrogen, oxygen, and nitrogen. The polyatomics that interfere with zinc isotopes (64, 66, 67, 68, and 70 amu) are limited to the combination of this matrix (H, O, and N), the plasma minerals (Na, Cl, S, K, P, and Ca) and argon (used as a source for the inductively coupled plasma). Therefore, in order to investigate possible interferences, solutions were prepared which contained only hydrogen, oxygen, and nitrogen except for the mineral elements cited.

Single-mineral solutions that contained the respective mineral found in the "actual human plasma" were prepared by dissolving each salt or diluting each acid in Milli-Q water. Nitric acid (1.2*M*) was prepared by diluting the concentrated nitric acid using Milli-Q water. Calcium car-

bonate (1250 mg) was dissolved by a few drops of nitric acid and the excess acid was evaporated by heating on a hot plate to obtain calcium nitrate. To start, 3600 μg Na/mL as sodium nitrate, 3300 μg Cl/mL as hydrochloric acid, 1200 μg S/mL as sulfuric acid, 189 μg K/mL as potassium nitrate, 141 μg P/mL as diammonium monohydrogen phosphate, and 99 μg Ca/mL as calcium nitrate were prepared. Finally, solutions containing one-tenth of each mineral concentration usually found in the representative human plasma were prepared in 0.12*M* nitric acid with 50 ng Y/mL as an internal standard. Single-mineral solutions contained only one mineral element—Na, Cl, S, K, P, or Ca. Solutions containing two different mineral elements were also prepared. The mixture of all minerals contained all six mineral elements (Na, Cl, S, K, P, and Ca).

Inductively Coupled Plasma–Mass Spectrometry

A VG PlasmaQuad-1, upgraded to PlasmaQuad-2 plus status (VG Instruments, Winsford, England, UK) ICP-MS instrument was used for all isotope ratio measurements. Each solution was aspirated and nebulized (Meinhardt concentric type) into the argon plasma (8000–6000 K temperature) via a peristaltic pump with a flow rate of approximately 1 mL/min. The yttrium (mass 89) internal standard was used to correct errors due to instrumental drifts during data acquisitions. Isotope ratio analyses were performed using the "Peak-Jump Acquire" IR data acquisition mode of the VG PlasmaQuad software. The peak-jump acquisition mode gave better relative standard deviations (RSD 0.2–0.3%) compared to the scan acquisition mode (2–4%) and the peak-jump mode is the highly recommended mode for IR measurements. The mass range scanned was 50–95 amu with 200 scan sweeps of 2048 channels, 160 μs dwell time per channel, and 200 peak jump sweeps with 10240 μs per peak jump sweep. These mass spectral acquisition parameters normally require 20 min acquisition time for 10 replicate measurements of each sample. Instrument control, methods procedures, and the data processing system, including calculations and statistics, were operated via a Compaq AT personal computer with version 3.2 of the VG PlasmaQuad software. All the four Zn isotope ratios ($^{67}Zn/^{64}Zn$, $^{67}Zn/^{66}Zn$, $^{67}Zn/^{68}Zn$, $^{67}Zn/^{70}Zn$) were measured in each sample. The mass discrimination among Zn isotopes was corrected by the frequent measurements of Zn standard solutions (125, 250, and 500 ng/mL) during the sequence of IR analysis.

Measurement of Polyatomic Interferents in the "Simulated Human Plasma" Solutions

After careful cleaning of the sampling/skimmer cones, torch, nebulizer, and the spray chamber, the various "simulated human plasma" mineral solutions prepared to quantify the ICP-MS interferents were

introduced into the argon plasma and the counts at the desired atomic mass units (64, 66, 67, 68, 70, and 89) were recorded using the "Peak-Jump Acquire" Isotope Ratio data acquisition mode as described earlier. The counts obtained at the desired atomic mass units were compared with the counts obtained from 250 ng Zn/mL and the equivalent concentrations of the interferents were calculated.

Calculations

Subtraction of the hydrogen peroxide mass spectral signal counts from each sample counts gave the blank-subtracted counts. Using the blank-subtracted signal counts, the four $^{67}Zn/^{64}Zn$, $^{67}Zn/^{66}Zn$, $^{67}Zn/^{68}Zn$, $^{67}Zn/^{70}Zn$ IR values were recalculated for each sample. The value obtained after subtraction of the baseline (zero time) IR from each IR value was divided by the natural Zn IR value to obtain the normalized IR (NIR) value. A data set of 4 normalized isotope ratios (67/64, 67/66, 67/68, and 67/70) × 163 time points × 2 treatments (extraction and nonextraction) obtained from 14 subjects after an iv dose of ^{67}Zn was subjected to statistical analysis. All statistical analyses were carried out using the SYSTAT5 (version 5.2.1) Macintosh software (SYSTAT Inc., Evanston, IL, USA).

RESULTS AND DISCUSSION

Inductively coupled plasma–mass spectrometry has become a powerful alternative for the determination of isotope ratio measurements along with other well-established techniques such as neutron activation analysis and thermal ionization and fast atom bombardment mass spectrometry. However, when biological material is analyzed by ICP-MS, potential interferences from polyatomic ions must be considered. These interfering polyatomic ions originate mainly from argon, nitrogen, and/or oxygen in combination with Na, S, Cl, and Ca, which are present at approximate concentration ranges of 3130–3370, 1120–1270, 2940–4120, and 92–109 mg/L in human serum, respectively (27). Zinc has five isotopes: 64, 66, 67, 68, and 70. The most abundant isotope, ^{64}Zn (48.9%), is interfered to a large extent by polyatomic ions containing sulfur, oxygen, and calcium.

Polyatomic Interferences During the IR Measurements of the "Simulated Human Plasma" Mineral Solutions

In order to accurately calculate the actual polyatomic background signals generated during the ICP-MS analysis of the digested plasma solutions, the various "simulated human plasma" mineral solutions were

Table 1
Equivalent Concentrations (ng/mL) of the Interferents in the Mineral Solutions to Naturally Occurring Zinc

	Mass number				
	64	66	67	68	70
Single mineral					
S	3.61	0.38	0.01	0.07	-1.45
Na	0.59	0.48	0.28	0.49	0.60
Cl	0.25	0.25	2.58	0.30	1.59
K	-0.08	-0.09	-0.15	-0.10	-0.47
P	-0.09	-0.11	-0.14	-0.11	-0.63
Ca	-0.08	-0.09	-0.15	-0.09	-0.58
Mixture of two minerals					
S - Na	5.53	0.55	0.22	0.10	0.00
S - Cl	5.08	0.65	0.94	0.29	6.82
S - K	4.19	0.17	-0.13	-0.19	-0.62
S - P	3.96	0.32	-0.02	-0.03	-0.58
S - Ca	3.45	0.13	-0.05	-0.15	-0.80
Na - Cl	1.02	0.63	1.00	0.65	13.35
Na - K	0.50	0.27	0.40	0.29	1.27
Na - P	0.74	0.38	0.67	0.40	2.15
Na - Ca	0.45	0.31	0.48	0.33	0.83
Cl - K	0.17	0.16	2.78	0.21	3.10
Cl - P	0.22	0.22	2.65	0.25	1.34
Cl - Ca	0.15	0.15	1.94	0.17	1.14
K - P	-0.08	-0.09	0.08	-0.08	-0.23
K - Ca	0.00	-0.01	0.11	0.00	-0.23
P - Ca	0.07	0.06	0.32	0.08	-0.23
Mixture of all minerals	6.66	0.90	0.94	0.39	8.51
Sum of the single minerals	4.19	0.82	2.43	0.57	-0.95

Each solution contains one-tenth of the mineral concentration(s) found in the representative human plasma.

S: 120 µg/mL S as H_2SO_4. Na: 330 µg/mL Na as $NaNO_3$. Cl: 360 µg/mL as HCl. K: 18.9 µg/mL K as KNO_3. P: 14.1 µg/mL as $(NH_4)_2HPO_4$. Ca: 9.9 µg/mL Ca as $Ca(NO_3)_2$.

All mineral solutions were prepared in 0.12*M* HNO_3.

subjected to the IR analyses of the routine ICP-MS instrumental conditions. Table 1 shows the equivalent contributions of the interferents in the mineral solutions to naturally occurring Zn. Investigations of the mineral solutions were limited to the single-mineral elements, and the mixture of two mineral elements and all mineral elements. The investigation of the interaction among three mineral elements or more were omitted because of the statistical difficulties. The mixture of all minerals was tested because it was the closest to the digested human plasma. Tenfold diluted solutions (in 0.12*M* nitric acid) were chosen because we utilized similar dilutions in the ongoing Zn nutritional study.

The 10-fold dilution of the digested human plasma contains approximately 100 ng Zn/mL. A careful study of the results from single-element

solutions in Table 1 indicates that the polyatomic interferences to ^{64}Zn by sulfur (3.61 ng/mL) and to ^{67}Zn by chlorine (2.58 ng/mL) alone are significant. On the other hand, the mixture of all mineral elements (S, Na, Cl, K, P, and Ca), which is approximately equivalent to the digested human plasma, largely interfered only with ^{64}Zn (6.66 ng/mL) and ^{70}Zn (8.51 ng/mL). However, the interferences to ^{66}Zn, ^{67}Zn, and ^{68}Zn are minimal—0.90, 0.94, and 0.39 ng/mL, respectively.

It is obvious from these results that interactions among mineral elements evoked the shift of the interferents from 67 to 70 atomic mass units. The mixture of Na–Cl, S–Cl, Cl–K, and Na–P evoked the interference with ^{70}Zn. The mixture of S–Cl and Na–Cl reduced the interference with ^{67}Zn. These results suggest that copresence of Na or S affects the chemical reaction of Cl in argon plasma, and the major interferent is shifted from $^{35}Cl^{16}O_2$ (atomic mass 67 coming from the Cl solution) to $^{35}Cl_2$. Table 2 summarizes the possible polyatomic species generated by the introduction of single and various combinations of mineral solutions to the inductively coupled argon plasma.

Comparison of Isotope Ratio Results from Human Plasma Samples—Extraction Versus Nonextraction

Isotope ratios were compared from Zn extracted (A) and nonextracted (B) human plasma specimens. Table 3 lists the range of normalized isotope ratios (NIRs) for the four isotope ratios chosen. When Zn is extracted from a sample, Zn isotopes 64, 66, and 68 can be used as a denominator isotope to calculate the normalized isotope ratio. However, the low abundance and counts of ^{70}Zn does not allow an accurate measurement of the normalized isotope ratio even after the extraction of Zn. As expected, all the NIR values were found to be the lowest after 9 d of intravenous administration of ^{67}Zn and highest at 5 min after injection. Negative values are irrational because all NIRs were obtained only after the administration of ^{67}Zn. The frequency of negative values for NIR was found to be very low; 2 out of 163 values (1.2%) for NIR-A $^{67}Zn/^{70}Zn$ and 7 out of 163 values (4.3%) for NIR-B $^{67}Zn/^{70}Zn$. Negative values were not observed for NIRs obtained from $^{67}Zn/^{66}Zn$ and $^{67}Zn/^{68}Zn$.

Figure 1 shows correlation plots of normalized isotope ratios of $^{67}Zn/^{68}Zn$ versus $^{67}Zn/^{66}Zn$ for extracted (A, $r^2 = 0.998$) and nonextracted (B, $r^2 = 0.992$) plasma samples. Only at very low NIR values do the data points tend to deviate from linearity for the nonextracted samples. The value of $r^2 = 0.992$ obtained for nonextracted samples is very close to $r^2 = 0.998$ for extracted samples and is acceptable for kinetics. Table 4 summarizes the correlations (r^2) between different NIRs obtained from extracted samples only using simple linear regression and double logarithmic (power function fitting) plots. As expected, the correlations

Table 2
Possible Polyatomics Generated by the Introduction of the Mineral Solutions to Inductively Coupled Argon Plasma

	Mass number				
	64	66	67	68	70
Single mineral					
S	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
Na	-	-	-	-	-
Cl	-	-	$^{35}Cl^{16}O_2$	-	-
K	-	-	-	-	-
P	-	-	-	-	-
Ca	-	-	-	-	-
Mixture of two minerals					
S - Na	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
S - Cl	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	$^{35}Cl_2$
S - K	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
S - P	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
S - Ca	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
Na - Cl	-	-	-	-	$^{35}Cl_2$
Na - K	-	-	-	-	-
Na - P	-	-	-	-	$^{23}Na^{31}P^{16}O$
Na - Ca	-	-	-	-	-
Cl - K	-	-	$^{35}Cl^{16}O_2$	-	$^{35}Cl_2$
Cl - P	-	-	$^{35}Cl^{16}O_2$	-	-
Cl - Ca	-	-	-	-	-
K - P	-	-	-	-	-
K - Ca	-	-	-	-	-
P - Ca	-	-	-	-	-
Mixture of all minerals	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	$^{35}Cl_2$
Sum of the single minerals	$^{32}S^{16}O_2$, $^{32}S_2$	-	$^{35}Cl^{16}O_2$	-	-

Each solution contains one-tenth of the mineral concentration(s) found in the representative human plasma.

S: 120 µg/mL S as H_2SO_4. Na: 330 µg/mL Na as $NaNO_3$. Cl: 360 µg/mL as HCl. K: 18.9 µg/mL K as KNO_3. P: 14.1 µg/mL as $(NH_4)_2HPO_4$. Ca: 9.9 µg/mL Ca as $Ca(NO_3)_2$.

are high for all the four isotopes, 64, 66, 67 and 68, because of the removal of the interfering polyatomic background ions during the extraction of Zn. Figure 2 shows the correlations of normalized isotope ratios for $^{67}Zn/^{68}Zn$ (A, $r^2 = 0.987$) and $^{67}Zn/^{66}Zn$ (B, $r^2 = 0.976$) for extracted versus nonextracted plasma samples.

Table 5 compares the normalized isotope ratios obtained from both the extracted (NIR-A) and nonextracted (NIR-B) samples using simple linear regression and double logarithmic (power function fitting) plots.

Table 3
The Range of Normalized Isotope Ratios

	"Extracted" Samples			
	67/64	67/66	67/68	67/70
Minimum	0.04	0.06	0.06	-0.43
Median	0.78	0.75	0.72	0.68
Maximum	14.33	13.67	12.91	12.89
	"Nonextracted" Samples			
	67/64	67/66	67/68	67/70
Minimum	-0.160	0.05	0.06	0.05
Median	0.53	0.71	0.75	0.69
Maximum	12.42	13.23	12.69	12.15

Minimum NIR was found 9 d after iv dose of ^{67}Zn.
Maximum NIR was found 5 min after iv ^{67}Zn administration.
Negative values are irrational because all NIRs were obtained after administration of ^{67}Zn.

NIR-B calculated from 67/68 and 67/66 agrees very well with NIR-A. As expected from the results of the detailed investigation of polyatomic interferences for ^{64}Zn and ^{70}Zn (Tables 1 and 2), the agreement between NIR-A and NIR-B calculated from 67/64 and 67/70 was poor. For $^{67}Zn/^{64}Zn$, $r^2 = 0.838$ and for $^{67}Zn/^{70}Zn$, $r^2 = 0.747$ (see Table 5). As a result of sulfur and oxygen polyatomic backgrounds at ^{64}Zn mass (mostly $^{32}S^{16}O_2$ and $^{32}S_2$) and shifting of the major interferent $^{35}Cl^{16}O_2$(atomic mass 67 coming from Cl in the solution) to $^{35}Cl_2$ (atomic mass 70), in combination with a very low natural abundance for ^{70}Zn, account for the poor agreement.

Ideally, extraction of zinc as a purification step appears desirable. The Zn extraction procedure, however, involves many steps, a deterrent for large numbers of samples because some steps are susceptible to contamination of Zinc from the environment. In summary, the regression analyses values (r^2, the slope a, and the intercept b) for NIR correlations from both the extraction and nonextraction methods show high correlations for $^{67}Zn/^{68}Zn$ and $^{67}Zn/^{66}Zn$. Such high correlations for nonextracted samples can be routinely achieved by (1) keeping the resolution of the mass spectrometer less than unity peak width (0.8–0.9 amu), (2) cleaning the skimmer and sampling cones, torch, and the nebulizer prior to analysis of each batch of samples, and (3) passing nitric acid (1%) followed by Milli-Q water between the samples until the ^{89}Y (internal standard) signal reaches below 200 counts (the rate meter reading approximately 2 at the 1-kHz setting).

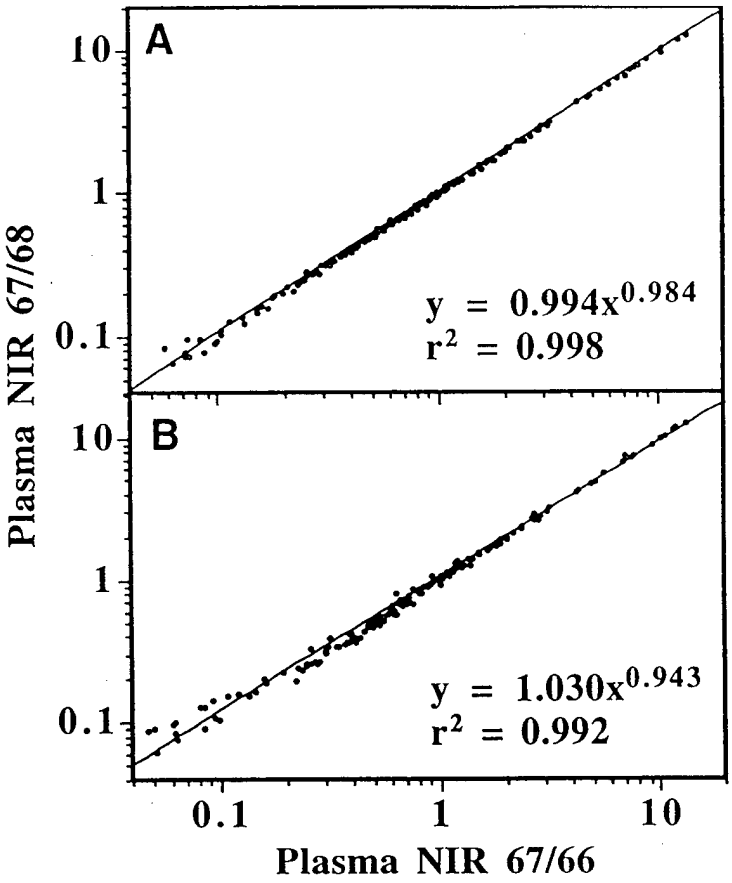

Fig. 1. Correlation plots of normalized isotope ratios of $^{67}Zn/^{68}Zn$ versus $^{67}Zn/^{66}Zn$ for extracted (A) and nonextracted (B) plasma samples.

Table 4
Correlations (r^2) Between Different NIRs Obtained from Extracted Samples Using Simple Linear Regression and Double Logarithmic (Power Function Fitting) Plots

	Simple Linear Regression Plot		
	67/66	**67/68**	**67/70**
67/64	0.996	0.994	0.887
67/66		0.999	0.889
67/68			0.802
	Double Logarithmic Plot		
	67/66	**67/68**	**67/70***
67/64	0.996	0.991	0.786
67/66		0.998	0.794
67/68			0.893

*Negative values were removed for calculation.

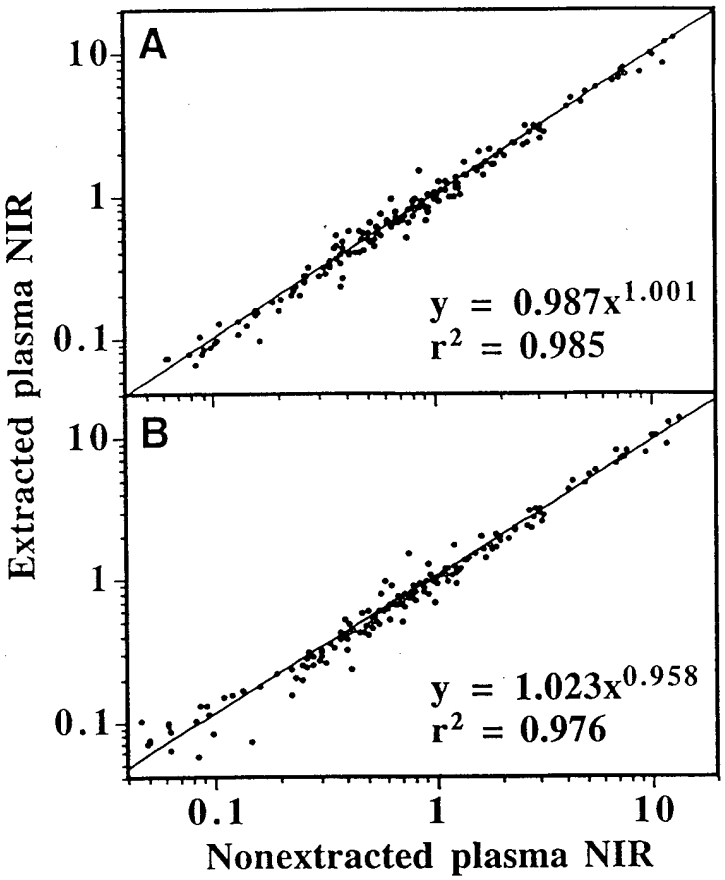

Fig. 2. Correlation plots of normalized isotope ratios for $^{67}Zn/^{68}Zn$ (A) and $^{67}Zn/^{66}Zn$ (B) for extracted versus nonextracted plasma samples.

Considering the possibility of isobaric interferences generated during the ionization processes of the digested plasma samples inside the inductively coupled plasma of the ICP-MS coupled with this detailed investigation indicate that $^{67}Zn/^{68}Zn$ and $^{67}Zn/^{66}Zn$ NIRs with the least possibility of polyatomic backgrounds obtained from nonextracted samples are sufficient for routine Zn kinetic analysis using a ^{67}Zn-enriched isotope. It should be pointed out that because the polyatomic backgrounds at atomic mass 67 are shifted to atomic mass 70, the nonextraction procedure may not be suitable for Zn kinetic analysis using ^{70}Zn-enriched stable isotope.

Table 5
Comparison of Normalized Zn Isotope Ratios (NIRs) Obtained from Extracted (A Batch, NIR-A) and Nonextracted (B batch, NIR-B) Samples Using Simple Linear Regression and Power Function Fitting (Double Logarithmic) Plots

Simple Linear Plot			
NIR	**r2**	**a**	**b**
67/64	0.838	0.059	0.175
67/66	0.983	0.985	0.040
67/68	0.985	0.964	0.035
67/70	0.747	0.907	0.132
Double Logarithmic Plot			
NIR	**r2**	**a**	**b**
67/64*	0.838	1.237	0.773
67/66	0.976	1.023	0.958
67/68	0.985	0.987	1.001
67/70*	0.747	0.966	0.903

Regression equation for the simple linear equation is NIR-A = *a* NIR-B + *b*, where *a* and *b* are the slope and the intercept, respectively. For perfect correlations, *a* should be equal to 1 and *b* should be zero.

Regression equation for the double logarithmic plot is NIR-A = *a* NIR-B power *b*. If both the values completely agree, then *a* and *b* should each be equal to 1.

*Negative values were removed because they do not allow fitting.

ACKNOWLEDGMENTS

We acknowledge the Department of the Army Medical Department (DAMD 17-95-C-5112) for financial support for the project. The administration of the ^{67}Zn stable isotope to subjects and collection of blood samples were conducted at the General Clinical Research Center, University of Texas Medical Branch at Galveston, funded by grant MO1 RR-00073 from the National Center for Research Resources, NIH, USPHS.

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QUARTERLY REPORT

1. Contract No.: DAMD17-95-C-5112 2. Report Date: 2/12/99

3. Reporting Period from: 9/23/98 to 12/22/98

4. PI: Harold H. Sandstead 5. Telephone No. (409) 772-4661

6. Institution: The University of Texas Medical Branch

7. Project Title: Repletion of Zinc and Iron deficiencies improves cognition of premenopausal women.

8. Current Staff, with percent effort of each on project:

Harold H. Sandstead	15%	Nancy W. Alcock	10 %
VM Sadagopa Ramanujam	25%	Hari H. Dayal	10 %
Norman G. Egger	90%	Michael Loftus	100 %
Renee Galloway	75%		

9. Contract expenditures to date (as applicable):

	This Qtr/Cumulative		This Qtr/Cumulative
Personnel:	$17,743/390,102	Travel:	$0/4,299
Fringe Benefits:	$5,020/91,043	Equipment:	$0/2,443
Supplies:	$5,012/95,273	Other:	$0/0

	This Qtr/Cumulative
Subtotal:	$27,775/583,160
Indirect Costs:	$19,756/289,978
Fee:	$0/0
TOTAL:	**$47,531/873,138**

10. Administrative and logistical matters.

a) During this quarter, 42 women contacted us by phone and expressed their interest (658 since the start of the study). Twenty (282 since the start of the study) potential subjects were screened by history, physical examination and laboratory assessment during an outpatient visit. Twelve of them qualified for the assessment of baseline zinc status assessment (119 since the start of the study). Thirteen subjects were enrolled in the intervention part of the study (95 since the start of the study). Twelve subjects completed the first phase of the intervention and started the second phase (71 since the start of the study). Five subjects completed the study (54 since the start of the study).

b) Enrollment continues to be very satisfactory. We have completed study of the 20 normal subjects. We are now concentrating all of our efforts on recruitment of the 60 low iron-low Zn subjects. About 25-30% of the women screened have serum ferritin concentrations <20ng/mL.

c) The administrative secretary hired last quarter left for a higher-ranking post. We have been without this support since December 11, 1998. Other secretarial staff has been very helpful. We hope to hire a replacement in the near future.

d) Our old ICP-MS has had recurrent problems the past quarter. The instability precluded our doing analysis of stable zinc.

e) We purchased a new ICP-MS. We are in the process of determining the best conditions for analysis.

11. Scientific progress.

a) This project is being done double-blind. Therefore there are no outcomes data to report.

b) Our manuscript "Polyatomics in Zinc Isotope Ratio analysis of Plasma samples by Inductively coupled Plasma-Mass Spectroscopy and Applicability of Nonextracted Samples for Zinc Kinetics" will appear in the March or April issue of the journal Biological Trace Elements.

c) Analysis white blood cell zinc was completed in 14 subjects All values were with in the reference range.

d) Analysis of plasma zinc, urine zinc, and hair zinc have been done at each stage of the project. Because of the double-blind design of the study we do not know the effect of the specific treatments on these indices.

QUARTERLY REPORT

1. Contract No.: DAMD17-95-C-5112 2. Report Date: 3/29/99

3. Reporting Period from: 12/23/98 to 3/22/99

4. PI: Harold H. Sandstead 5. Telephone No. (409) 772-4661

6. Institution: The University of Texas Medical Branch

7. Project Title: Repletion of Zinc and Iron deficiencies improves cognition of premenopausal women.

8. Current Staff, with percent effort of each on project:

Harold H. Sandstead	15%	Nancy W. Alcock	10 %
VM Sadagopa Ramanujam	25%	Hari H. Dayal	10 %
Norman G. Egger	90%	Michael Loftus	100 %
Renee Galloway	75%		

9. Contract expenditures to date (as applicable):

	This Qtr/Cumulative		This Qtr/Cumulative
Personnel:	$13,259/403,361	Travel:	$0/4,299
Fringe Benefits:	$4,103/95,056	Equipment:	$0/2,443
Supplies:	$5,902/101,175	Other:	$0/0

	This Qtr/Cumulative
Subtotal:	$23,174/606,334
Indirect Costs:	$11,587/301,565
Fee:	$0/0
TOTAL:	**$34,761/907,899**

10. Administrative and Logistical Matters

a) During this quarter, 50 women contacted us by phone and expressed their interest (708 since the start of the study). Thirty-seven (319 since the start of the study) potential subjects were screened by history, physical examination and laboratory assessment during an outpatient visit. Eight of them qualified for the assessment of baseline zinc status assessment (127 since the start of the study). Eight subjects were enrolled in the intervention part of the study (103 since the start of the study). Seven subjects completed the first phase of the intervention and started the second phase (78 since the start of the study). Ten subjects completed the study (64 since the start of the study). Twenty-one subjects are currently at some phase of the intervention study. Completion by 16 of them will meet our subject needs. Thus, we have a redundancy of 5 subjects. We will use them to replace dropouts. If no dropouts occur, we will include findings from these individuals in the database, this increasing the power of our observations.

b) Enrollment was so satisfactory we stopped recruiting new subjects.

c) A new administrative secretary was appointed.

d) The new ICP-MS is up and running. Has had recurrent problems the past quarter. The instability precluded our doing analysis of stable zinc.

11. Scientific Progress

a) This project is being done double-blind. Therefore there are no outcomes data to report.

b) Our manuscript "Polyatomics in Zinc Isotope Ratio analysis of Plasma samples by Inductively coupled Plasma-Mass Spectroscopy and Applicability of Nonextracted Samples for Zinc Kinetics" will appear in the April issue of the journal Biological Trace Elements.

c) Analysis white blood cell zinc was completed in 5 subjects. All values were with in the reference range.

d) Analysis of plasma zinc, urine zinc, and hair zinc is continuing.

QUARTERLY REPORT

1. Contract No.: DAMD17-95-C-5112 2. Report Date: 6/30/99

3. Reporting Period from: 03/22/99 to 6/22/99

4. PI: Harold H. Sandstead 5. Telephone No. (409) 772-4661

6. Institution: The University of Texas Medical Branch

7. Project Title: Repletion of Zinc and Iron deficiencies improves cognition of premenopausal women.

8. Current Staff, with percent effort of each on project:

Harold H. Sandstead	15%	Nancy W. Alcock	10 %
VM Sadagopa Ramanujam	25%	Hari H. Dayal	10 %
Norman G. Egger	90%	Michael Loftus	100 %
Drue E. Pean	75%		

9. Contract expenditures to date (as applicable):

	This Qtr/Cumulative		This Qtr/Cumulative
Personnel:	$9327/412,688	Travel:	$0/4,299
Fringe Benefits:	$2681/97,737	Equipment:	$0/2,443
Supplies:	$4768/105,944	Other:	$0/0

	This Qtr/Cumulative
Subtotal:	$16,776/623,110
Indirect Costs:	$ 6611/308,176
Fee:	$0/0
TOTAL:	**$23,387/931,286**

10. Administrative and logistical matters.

a) During this quarter, no women contacted us by phone and expressed their interest (708 since the start of the study). No potential subjects were screened (a total of 319 were screened since the start of the study). One subject completed assessment of zinc status (a total of 128 volunteers have completed this part of the study). Eight subjects were enrolled in the intervention trial (111 since the start of the study), and 12 volunteers completed the first phase of the intervention (90 since the start of the study). Eight subjects completed the study (72 since the start of the study). Two subjects are part of the way through the first phase of the intervention and 10 are part way through the second phase of the intervention. Therefore there are more than enough subjects 'in the pipeline' to fill all cells of the intervention trial.

b) A first year medical student, Anika Bell did an elective research project with us. Following University guidelines we paid her a small stipend during her 8 week research experience. She is seeking continued support though the University "work-study" program to continue her work.

11. Scientific progress.

a) This project is double blind. Therefore data on functional outcomes are not available.

b) We (VMS Ramanujam, K Yokoi, NG Egger, HH Dayal, NW Alcock, and HH Sandstead) published "Polyatomics in Zinc Isotope Ratio analysis of Plasma samples by Inductively coupled Plasma-Mass Spectroscopy and Applicability of Nonextracted Samples for Zinc Kinetics." in the Journal Biological Trace Elements 1999; 68: 143-58.

c) Analysis white blood cell zinc is continuing.

d) Analysis of plasma, urine, and hair zinc is continuing.

e) The student presented her work at a 'Student Research Day,' June 17, 1999. She reported relationships between trace metal content of hair from 25 subjects, 13 in the highest quartile for hair zinc (>291 μg/g hair) and 12 in the lowest quartile for hair zinc (<113 μg/g hair) from subjects at baseline. Hair was analyzed by inductively coupled plasma-mass spectrometry and atomic absorption spectrometry. Pearson correlation's found 25 significantly related pairs of elements in the low zinc group and 7 significantly related pairs of elements in the high zinc group. Serum ferritin concentrations were related to the concentration of iron in hair ($R^2 = 0.23$, $p < 0.01$). Manganese concentration in hair was related to the zinc concentration in hair ($R^2 = 0.26$, $p < 0.009$). Frequency of consumption of mushrooms and of cooked hot cereals were directly associated ($p = 0.01$ and $p = 0.03$, respectively) with

hair zinc concentrations. As far as we are aware, these findings are unique. These data provide the basis for further examination of these relationships.

QUARTERLY REPORT

1. Contract No.: DAMD17-95-C-5112 2. Report Date: 6/30/99

3. Reporting Period from: 06/22/99 to 9/22/99

4. PI: Harold H. Sandstead 5. Telephone No. (409) 772-4661

6. Institution: The University of Texas Medical Branch

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VM Sadagopa Ramanujam	25%	Hari H. Dayal	10 %
Norman G. Egger	90%	Michael Loftus	100 %
Drue E. Pean	75%		

9. Contract expenditures to date (as applicable):

	This Qtr/Cumulative		This Qtr/Cumulative
Personnel:	$4859/417,547	Travel:	$0/4,299
Fringe Benefits:	$1197/98,934	Equipment:	$0/2,443
Supplies:	$6185/112,129	Other:	$0/0

	This Qtr/Cumulative
Subtotal:	$12,241/635,352
Indirect Costs:	$ 7897/316,073
Fee:	$0/0
TOTAL:	**$20,138/951,425**

10. Administrative and logistical matters

a) Collection if neuropsychological data was completed. All treatment cells were filled.

b) The neuropsychological data were sent to our collaborator James Penland, PhD, Research Psychologist, USDA ARS Grand Forks Human Nutrition Center (GFHNRC), Grand Forks, ND for analysis. We will send him duplicates of the other data. We will utilize the biostatistical resources of the GFHNRC. Data entry will be done in a timely manner by graduate students hired on a by the hour basis. This will result in significant cost savings.

11. Scientific progress

a) Results of the neuropsychological studies will become available over the next several months.

b) Ten subjects blood plasma were analyzed for stable isotope concentrations by ICP-MS. Data are being used for calculating Zn kinetics. Samples from 23 subjects remain to be analyzed.

c) Hair samples from 11 women were analyzed for 21 elements by ICP-MS. This aspect of the project is just beginning.

d) An abstract was presented at the 1999 Experimental Biology Meetings in Washington, DC.

DETERMINATION OF THE RAPIDLY EXCHANGEABLE ZINC POOL IN HUMANS BY RANDOME URINE SPECIMEN. K Yokoi, NG Egger, VMS Ramanujam, NW Alcock, HH Dayal, and HH Sandstead